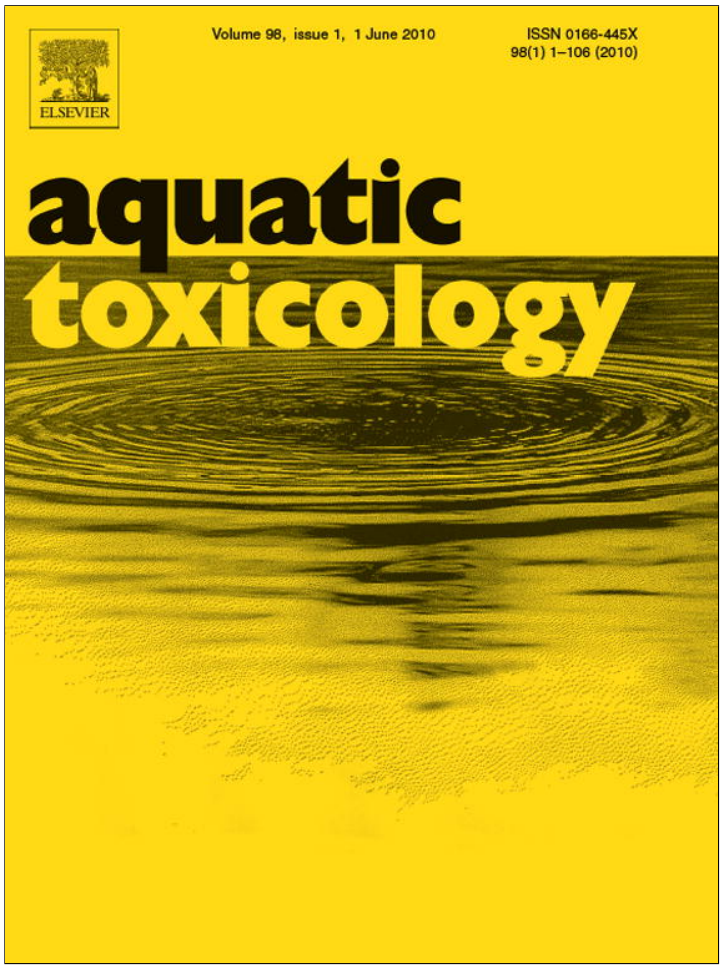

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Early temporal effects of three thyroid hormone synthesis inhibitors in *Xenopus laevis*

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ABSTRACT

Thyroid axis disruption is an important consideration when evaluating risks associated with chemicals. Bioassay methods that include thyroid-related endpoints have been developed in a variety of species, including amphibians, whose metamorphic development is thyroid hormone (TH)-dependent. Inhibition of TH synthesis in these species leads to developmental delay, and assays designed to capture these effects take several weeks to complete. In an effort to develop a shorter term approach, the early responses of various endpoints were evaluated in *Xenopus laevis* throughout 8 d of exposure to three TH synthesis inhibitors: methimazole (100 mg/L), 6-propylthiouracil (6-PTU) (20 mg/L), and perchlorate (4 mg/L). Endpoints included thyroid gland histology and cell numbers, circulating TH concentrations, and thyroidal TH and associated iodo-compounds. Thyroidal 3,5-diodo-L-tyrosine (DIT) and thyroxine (T4) were significantly reduced from day 2 onward by all three chemicals, while 3-monoiodo-L-tyrosine (MIT) was significantly reduced by methimazole and perchlorate, but not by 6-PTU. These reductions were the earliest indicators of TH synthesis inhibition. Histological effects were apparent on day 4 and became more exaggerated through day 8. However, reductions in circulating T4 and increases in thyroid gland cell numbers were not apparent until day 6. Reductions of thyroidal MIT, DIT, and T4 and circulating T4 are indicative of inhibitory effects of the chemicals on TH synthesis. Changes in thyroid histology and cell number represent compensatory effects modulated by circulating TSH. These observations establish a basis for the development of short term amphibian-based methods to evaluate thyroid axis effects using a suite of diagnostic endpoints.

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1. Introduction

Assessing the impact of chemicals on thyroid function is an important component of a comprehensive screening program for endocrine disruption (DeVito et al., 1999). Several chemicals have been shown to alter normal thyroid function (Brucker-Davis, 1998) and an assortment of tests has been developed in a variety of vertebrate species to assess thyroid disruption using both *in vivo* and *in vitro* approaches (Zoeller and Tan, 2007). The relatively conservative nature of many components of the hypothalamic–pituitary–thyroid (HPT) axis among vertebrates suggests that extrapolation of chemical effects among species may be feasible (Zoeller and Tan, 2007). The amphibian HPT axis is a well studied system (Brown and Cai, 2007; Dodd and Dodd, 1976; Fort et al., 2007) with unique characteristics that make it amenable to bioassay procedures. For example, metamorphic development of many anurans is a thyroid hormone (TH)-dependent process that can be inhibited by exposure to chemicals which inhibit TH synthesis. Based on this knowledge, metamorphic development of *Xenopus laevis* has been proposed as a potential model system upon which a bioassay for thyroid hormone disruption could be developed (Degitz et al., 2005; Opitz et al., 2005; U.S. Environmental Protection Agency, 1998). In response to this proposal, a testing protocol has been developed which exposes premetamorphic larvae at the hind limb bud stage to a test chemical for 21 d and relies on thyroid gland histology and morphological indicators of development as its primary endpoints (OECD, 2009).

Thyroid gland histology is the most sensitive and diagnostic endpoint considered in the 21-d protocol. Thyroid glands of *X. laevis* exposed to TH synthesis inhibitors exhibit follicular cell hyperplasia and hypertrophy, alterations in colloid, and glandular growth; changes generally associated with the compensatory mechanisms which are thought to be modulated by increased circulating thyroid stimulating hormone (TSH) concentrations in response to declin-

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ing TH concentrations in the blood. These changes occur at TH synthesis inhibitor concentrations below those that result in the retardation of metamorphic development and their expression is relatively rapid, being clearly observable following 8 d of exposure to some chemicals (Degitz et al., 2005; Tietge et al., 2005). The changes observed in *X. laevis* thyroid glands are consistent with those observed in other species exposed to thyroid synthesis inhibitors (Hooth et al., 2001; Capen, 1994) suggesting further that this model system may be representative of other vertebrate thyroid systems.

The value of this assay, like any bioassay approach, is largely determined by the duration and cost of the test as compared to the diagnostic information gained. To improve the value of this model system, a shorter bioassay with more diagnostic endpoints is desirable. In order to achieve that goal, the early temporal responses of several endpoints associated with thyroid axis disruption need to be evaluated and compared to the results obtained through longer term studies. Consistent with this approach, early temporal histological and selected transcriptional responses of *X. laevis* larvae exposed to perchlorate and ethylenethiourea (ETU) were recently evaluated (Opitz et al., 2009). The histological responses observed in that study following exposure to perchlorate and ETU were similar to those noted above, including: follicular cell hyperplasia and hypertrophy, diffuse glandular hypertrophy, and alterations in follicular size. The temporal responses of several thyroidal genes were also evaluated and three genes associated with TH regulation and synthesis, thyroid stimulating hormone receptor (TSHr), sodium iodide symporter (NIS), and thyroperoxidase (TPO), were significantly upregulated following 3–5 d of exposure to each chemical. These results support the concept that a shorter term assay is feasible.

The objective of this study is to further evaluate the early temporal responses of additional endpoints related to TH synthesis inhibition besides thyroid gland histology and expression of selected genes. These endpoints include circulating T4 and T3 as indicators of overall thyroid function, thyroid gland T4, MIT, and DIT as indicators of TH synthesis, thyroid histology as an indicator of TSH-mediated compensation, and thyroid cell numbers as an alternate indicator of hyperplasia associated with compensation. These endpoints were evaluated throughout 8 d of exposure to three different chemicals known to inhibit TH synthesis: perchlorate, 6-propylthiouracil (6-PTU), and methimazole. These studies were initiated with prometamorphic organisms, a stage which coincides with the onset of thyroid gland function and normally increasing TH concentrations in the blood (Dodd and Dodd, 1976; LeLoup and Buscaglia, 1977). Perchlorate, 6-PTU, and methimazole were each tested at single concentrations of 4, 20, and 100 mg/L, respectively. These concentrations were selected based on previous studies which demonstrated that these were maximally effective concentrations in terms of metamorphic inhibition.

2. Materials and methods

2.1. Biological procedures

X. laevis tadpoles used for the exposures were obtained from an in-house culture. Reproduction was induced with human chorionic gonadotropin injections and the resultant embryos were held in clean water at 21 °C. Tadpoles were fed a mixture of TetraFin® (Tetra Sales, Blacksburg, VA, USA), Spirulina algae discs (The Wardley Corporation, Secaucus, NJ, USA), Silver Cup Trout Starter (Nelson & Sons Inc., Murray, UT, USA) and <24 h old live brine shrimp (Bio-Marine® Brand, Bio-Marine Inc., Hawthorne, CA, USA). The TetraFin®, algae discs, and trout starter were all blended in Lake Superior water prior to feeding. Tadpoles in all tanks were fed twice/day Monday through Friday and a double amount once/day on the weekend. After 21 d, tadpoles were netted from the tanks, anesthetized using 100 mg/L of MS-222 buffered with 200 mg/L of sodium bicarbonate, and the developmental stage of each tadpole was determined according to Nieuwkoop and Faber (NF) (1994). Tadpoles were placed in clean water to recover from anesthesia. After recovery, the tadpoles at stage NF54 were randomly distributed to the exposure tanks.

2.2. Exposure

Three different studies were conducted using the same exposure protocol to collect the data presented herein. Thyroid gland histology was analyzed from a study conducted in October 2004. Circulating TH concentrations and thyroid gland MIT, DIT, and T4 concentrations were measured from a study conducted in February 2007. Thyroid cell number data were measured from a study conducted in July 2007. No mortality or other overt signs of toxicity were observed in any of the studies. In each study, there were four replicate tanks of one exposure concentration for each chemical and controls. The water flow rate to each 4.0 L tank was 25 mL/min which resulted in 9 tank volume additions per day. Exposures were conducted at 21 °C in Lake Superior water which underwent ultraviolet light sterilization. The photoperiod was 12 h light:12 h dark. Exposure tanks were immersed in a water bath system to maintain temperature uniformity.

All test chemicals (methimazole, 6-PTU, and sodium perchlorate) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Stock solutions for methimazole and sodium perchlorate were made in a 19 L glass carboy by dissolving each chemical in water using a stir plate and a magnetic stir bar. Stock solutions for 6-PTU were made in a 19 L glass carboy using a high speed top stirrer to dissolve it in water at 40 °C. Nominal aqueous chemical concentrations of methimazole, 6-PTU, and perchlorate were 100 mg/L, 20 mg/L, and 4 mg/L, respectively. Exposure concentrations of each chemical in each test were analytically verified by either HPLC-DAD (methimazole and 6-PTU) or anion chromatography (perchlorate). Actual concentrations were within 5% of the nominal values for all chemicals in all tests and concentrations did not change throughout each study (data not shown).

2.3. Tissue sampling

Prior to any tissue sampling, larvae were anesthetized with 150 mg/L MS-222. For serum collection, larvae were placed in a dry dissection tray and the pericardial membrane was carefully pulled aside to expose the aorta. Excess pericardial fluid was then removed by blotting with filter paper. The aorta was severed for blood collection using micro-surgical scissors and whole blood was collected using either 10 or 25 μL capillary tubes. Blood was allowed to clot on ice for 4 h and was subsequently centrifuged at 13,700 × *g* for 2 min in a serological centrifuge. Serum was transferred to vials and stored at −80 °C until analysis by HPLC/ICP-MS. Serum samples from four individuals were pooled from each replicate tank at 1, 2, 4, and 6 d, yielding a serum sample size of 4 for each chemical at every time point.

Thyroid glands used for MIT, DIT, and T4 analysis were dissected with fine tip forceps from larvae held in cold L-15 media. The free glands were transferred to vials and stored at −80 °C until processing for analysis by HPLC/ICP-MS. Paired glands from four individuals were pooled from each replicate tank at 1, 2, 4, and 6 d, yielding a sample size of 4 for each chemical at every time point.

Anesthetized larvae were decapitated and head sections were fixed in Davidson's fixative for 48 h. The head samples were rinsed in water and stored in 10% neutral buffered formalin. The samples were dehydrated in a graded ethanol series and embedded in paraffin using standard histological procedures. Head samples were

placed in cassettes with the cut side down so that the heads were sectioned in a transverse plane. Five step sections (approximately 30 μm apart) were cut from each paraffin block, 30–50 μm after the sectioning plane had transected both thyroid glands. Two serial sections of each step were placed on each of five slides. The slides were stained with hematoxylin and eosin. Two slides were selected for evaluation of the thyroid glands of each larva. The first slide was selected on the basis of adequate transverse sections of both thyroid glands, and the second slide was two steps away from the first, if possible. Only one of the two serial sections on the slides was evaluated. Observations were made on the status of thyroid follicles, follicular cells, the colloid, and overall size of the gland, consistent with Grim et al. (2009). Microscopic findings for thyroid glands from each organism were graded from 1 to 5 depending upon severity (1 = minimal, 2 = slight/mild, 3 = moderate, 4 = moderately severe, and 5 = severe/high).

Thyroid cell numbers were quantified using the CyQUANT® Cell Proliferation Assay Kit (Invitrogen C7026) (Invitrogen Corp., Carlsbad, CA, USA). This fluorometric DNA assay was calibrated by measuring the fluorescence associated with thyroid cell suspensions of differing cellular densities. For calibration curve development, thyroid cell suspensions were prepared by pooling about 100 glands removed from non-exposed *X. laevis* larvae (NF stages 57–62) from general culture. The glands were initially transferred into Dulbecco's phosphate buffered saline without calcium or magnesium. The pooled tissue was digested in trypsin–EDTA solution at 37 °C, dispersed by pipette, and strained through 50 μm mesh to yield cell suspensions that were quantified using a hemacytometer. The cell suspensions were then frozen at −80 °C until DNA analysis. Samples were thawed at room temperature, brought up to final volumes in a cell lysis buffer provided in the CyQUANT® assay kit, then homogenized by sonication. Samples were treated with RNase A (QIAGEN Inc., Valencia, CA, USA) and DNA was quantified based on the CyQUANT® assay kit instructions using fluorescence measured on a microplate reader (Bio-Tek Instruments FLx800) (BioTek Instruments, Inc., Winooski, VT, USA). A standard curve relating fluorescence to thyroid cell density was developed in order to determine the amount of DNA per thyroid follicular cell. One thyroid gland was randomly sampled from five tadpoles from each replicate tank ($n = 4$) of each chemical at 2, 3, 4, 5, and 6 d. Thyroid glands ($n = 5$) from each replicate were pooled in L-15 media, frozen at −80 °C, and analyzed for DNA, as above. Cell numbers were calculated for each pool of glands using 7.7 pg DNA/cell from the previously established standard curve. Mean thyroid cell numbers were calculated using the four pooled replicate samples from each chemical at each time point.

2.4. HPLC/ICP-MS analysis

Thyroid hormone and related iodinated species were analyzed using high performance liquid chromatography (HPLC) and inductively coupled plasma mass spectrometry (ICP/MS) according to methods similar to those of Takatera and Watanabe (1993) and Michalke et al. (2000). The system consisted of an HPLC (Agilent Model 1100, Agilent Technologies, Inc., Santa Clara, CA, USA) interfaced with an ICP/MS (Agilent Model 7500CE). The HPLC module included a capillary binary pump system, a vacuum degasser, a refrigerated auto-sampler, and temperature controlled column compartment. Chromatographic separation was achieved by using a Phenomenex (R) Synergy Hydro RP C18 column (2 mm ID × 50 mm, 4um particle size) (Phenomenex, Inc., Torrance, CA, USA). Two mobile phases (A: 1% methanol with 25 mM formic acid; B: 90% methanol with 25 mM formic acid) were used in a 35 min gradient program at a flow of 450 μL/min. HPLC eluent was transferred directly to the ICP-MS via capillary peek tubing. The ICP-MS system was equipped with a micromist nebulizer, a shielded torch, and platinum cones. Oxygen (5%) was added to the plasma to combust the methanol in the mobile phases, thereby preventing carbon deposition at the cones. Samples were analyzed for iodine by monitoring mass 127.

Thyroid gland samples were thawed at room temperature, homogenized by sonication, and proteolytically digested using pronase (Sigma–Aldrich P8811) (Sigma–Aldrich, St. Louis, MO, USA) at a concentration of 10 mg/mL (approximately 40 units/mL) in phosphate buffered saline at pH 7.4 for 4 h at 37 °C, based on methods by Jansen et al. (1991). Samples were then acidified with 10 mM phosphoric acid buffer (pH 2.6) to a concentration of 35% (v/v) and centrifuged 5 min at 20,800 × *g*. Supernatant was transferred to new vials and stored at 4 °C overnight or until analysis by HPLC/ICP-MS. Serum samples were thawed at room temperature, diluted 1:10 with mobile phase A, and analyzed without further treatment. Both sample types were analyzed using a 100 μL injection. Thyroidal iodo-compounds are reported as pg/organism, whereas circulating T4 is reported as nM in the serum.

2.5. Statistics

Thyroid gland MIT, DIT, and T4, circulating T4, and thyroid gland cell number data were evaluated using two way analysis of variance. Multiple pairwise comparisons of treatments to controls were done using the Holm-Sidak test. Non-normal data were transformed using a log transformation. Results are reported using non-transformed data. All comparisons were considered significant at $p \leq 0.05$.

3. Results

3.1. Circulating

Circulating T4 and T3 were measured on exposure days 1, 2, 4, and 6. Circulating T4 concentrations (mean ± standard deviation) in the experimental controls increased from 0.196 ± 0.136 nM on day 1 to 0.779 ± 0.106 nM on day 6 (Fig. 1). During this interval, larvae developed from stage NF54 to NF56–NF57. Methimazole, perchlorate, and 6-PTU significantly reduced circulating T4 concentrations to 46, 54, and 49% of control concentrations, respectively, on day 6, but no significant effects were observed at the earlier time points except for a transient reduction by 6-PTU on day 2 (Fig. 1). T3 was not quantifiable in the serum because it was at or below detection limits of the method (0.015 nM).

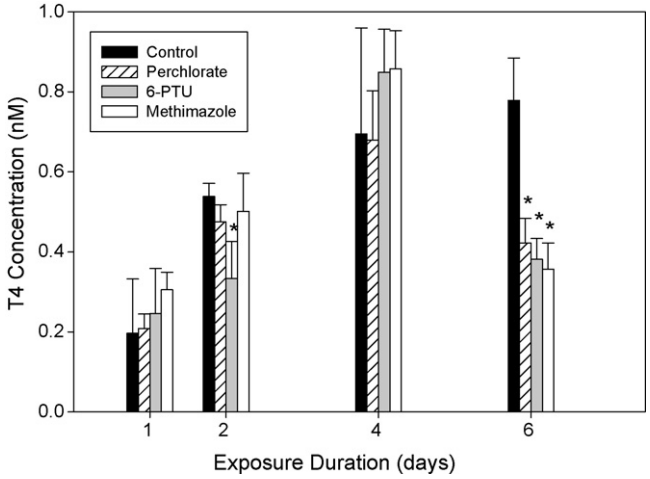

Fig. 1. Circulating T4 concentrations (nM) in NF54 *X. laevis* larvae exposed to perchlorate (4 mg/L), 6-PTU (20 mg/L), and methimazole (100 mg/L) for 1, 2, 4, and 6 d. Asterisks indicate significant difference from the control ($p \leq 0.05$).

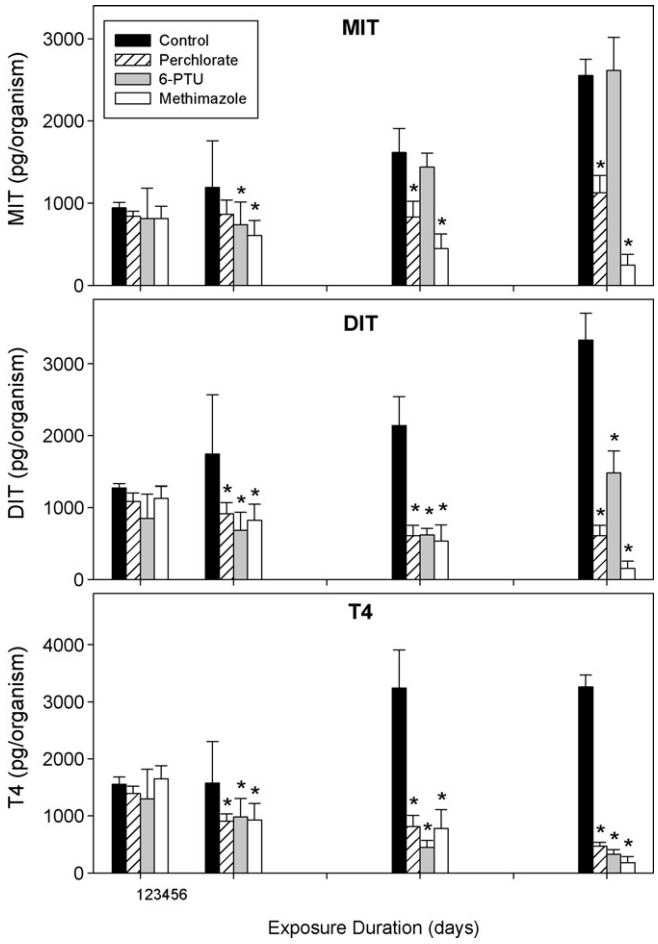

Fig. 2. Thyroidal MIT, DIT, and T4 mass (pg/organism)) in NF54 *X. laevis* larvae exposed to perchlorate (4 mg/L), 6-PTU (20 mg/L), and methimazole (100 mg/L) for 1, 2, 4, and 6 d. Asterisks indicate significant difference from the control ($p \leq 0.05$).

3.2. Thyroid gland MIT, DIT

Thyroid gland MIT, DIT, and T4 were measured on exposure days 1, 2, 4, and 6. The mass/organism of all three iodocompounds increased in the experimental controls throughout the study. Mean MIT concentrations rose from 942 ± 65 pg/organism on day 1 to 2550 ± 201 pg/organism on day 6. Mean DIT concentrations rose from 1279 ± 49 pg/organism on day 1 to 3333 ± 375 pg/organism on day 6. And, mean T4 concentrations rose from 1557 ± 123 pg/organism on day 1 to 3259 ± 208 pg/organism on day 6. Perchlorate exposure significantly reduced MIT concentrations beginning on day 4 and DIT and T4 concentrations beginning on day 2. Perchlorate exposure for 6 d reduced MIT, DIT, and T4 concentrations to 44, 18, and 14% of the time-matched control values, respectively (Fig. 2). 6-PTU exposure resulted in a transient significant reduction of MIT on day 2, but had no affect on glandular MIT throughout the remainder of the study. 6-PTU did result in significant reductions of thyroidal DIT and T4 compared to the controls beginning on day 2. These reductions continued through day 6 when DIT and T4 concentrations were reduced to 44 and 10% of the time-matched control values, respectively (Fig. 2). Methimazole exposure significantly reduced thyroidal MIT, DIT, and T4 beginning on day 2 and the magnitude of these reductions increased through time. Methimazole exposure for 6 d reduced thyroidal MIT, DIT and T4 to 10, 4, and 6% of the time-matched control values, respectively. Similar to the serum, T3 was not quantifiable in the thyroid gland samples due to low abundance.

3.3. Thyroid histopathology

Thyroid histology was evaluated on exposure days 2, 4, 6, and 8 based on previously described criteria (Grim et al., 2009). Thyroid glands of control frogs were characterized by colloid-filled follicles lined by simple cuboidal follicular cells. Minimal follicular cell hyperplasia was the only observation in the thyroid glands of experimental controls and occurred in only one organism at exposure day 2, in all organisms at days 4 and 6, and in all but one organism at day 8 (Table 1). This level of hyperplasia is considered the normal baseline condition indicative of normal glandular growth.

Exposure to all three chemicals resulted in follicular cell hyperplasia in excess of that observed in the controls, follicular cell hypertrophy, decreased colloid, and diffuse hypertrophy of the thyroid gland. In general, these effects were different from the experimental controls beginning on exposure day 4. The prevalence and severity of these effects increased throughout the exposure and were maximal in all cases on exposure day 8. In addition, exposure to 6-PTU resulted in a combination of selected follicular enlarge-

Table 1
Incidence and mean severity of thyroid gland histopathological observations for control, methimazole, perchlorate, and 6-propylthiouracil treated NF54 *X. laevis* larvae exposed for 2, 4, 6, and 8 d. Sample sizes are 5 organisms for each treatment at each time. SD indicates standard deviation.

Observation	Measurement	Control				Methimazole				Perchlorate				6-PTU			
		2 d	4 d	6 d	8 d	2 d	4 d	6 d	8 d	2 d	4 d	6 d	8 d	2 d	4 d	6 d	8 d
Follicular	Incidence	1	5	5	4	4	5	5	5	4	5	5	5	4	5	5	5
Cell	Mean severity	0.20	1.2	1.0	0.80	0.80	1.60	2.60	4.00	0.80	1.40	2.00	2.60	0.80	1.60	1.40	2.80
Hyperplasia	SD severity	0.45	0.45	0.00	0.45	0.45	0.55	0.89	0.00	0.45	0.55	0.00	0.55	0.45	0.55	0.55	0.45
Follicular	Incidence	0	0	0	0	0	3	4	5	0	5	5	5	1	4	3	5
Cell	Mean severity	–	–	–	–	–	1.20	2.40	3.40	–	2.00	3.00	3.60	0.20	1.20	0.80	2.40
Hypertrophy	SD severity	–	–	–	–	–	1.10	1.34	0.55	–	0.00	0.00	0.55	0.45	0.84	0.84	1.52
Decreased	Incidence	0	0	0	0	0	2	4	5	0	1	2	5	3	3	3	5
Colloid	Mean severity	–	–	–	–	–	0.80	2.00	4.00	–	0.40	0.80	3.40	0.80	0.80	0.60	3.00
	SD severity	–	–	–	–	–	1.30	1.58	0.71	–	0.89	1.30	0.89	0.84	0.84	0.55	0.71
Diffuse	Incidence	0	0	0	0	0	0	1	4	0	0	0	2	0	0	1	4
Hypertrophy	Mean severity	–	–	–	–	–	–	0.40	2.20	–	–	–	0.80	–	–	0.40	2.40
	SD severity	–	–	–	–	–	–	0.89	1.48	–	–	–	1.10	–	–	0.89	1.52
Follicular	Incidence	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5
Enlargement	Mean severity	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2.40
	SD severity	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.55

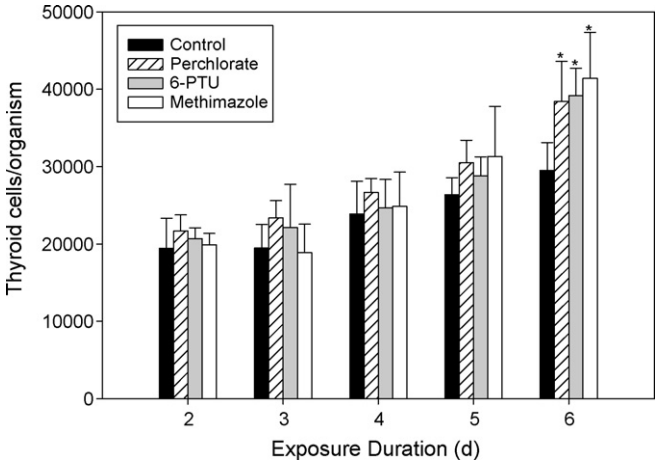

Fig. 3. Thyroid cell numbers (thyroid cells/organism) in NF54 *X. laevis* larvae exposed to perchlorate (4 mg/L), 6-PTU (20 mg/L), and methimazole (100 mg/L) for 2, 3, 4, 5, and 6 d. Asterisks indicate significant difference from the control ($p \leq 0.05$).

ment and collapse due to decreased colloid on exposure day 8 (Table 1).

3.4. Thyroid cell numbers

Thyroid cell numbers were determined on exposure days 2, 3, 4, 5, and 6. Thyroid cell numbers in the experimental controls increased significantly throughout the study from 1.95×10^4 cells/organism at NF54 on day 2 to 2.95×10^4 cells/organism at NF57 on day 6 (Fig. 3). There were no significant differences in thyroid cell numbers between any of the treatments and the controls for the first 5 d of exposure. However, 6 d of exposure to perchlorate, 6-PTU, and methimazole resulted in significant increases in thyroid cell numbers to 3.84×10^4, 3.92×10^4, and 4.14×10^4 cells/organism, respectively.

4. Discussion

The results of this study demonstrate that significant early temporal responses in several thyroid-related endpoints can be detected in *X. laevis* following exposure to three different thyroid hormone synthesis inhibitors. These responses are summarized in Table 2 which indicates the magnitude of each response as a percentage of the time-matched control or the prevalence of each response (where appropriate) in comparison to the time-matched controls. These data show that all three test chemicals rapidly inhibited TH synthesis in the thyroid gland, which resulted in reduced circulating T4 concentrations and compensatory responses of the thyroid as determined by histological analysis and an evaluation of thyroid cell numbers. These responses are valuable as potential endpoints in short term assays for the thyroid disruption and as endpoints of interest regarding the temporal dynamics of thyroid gland function.

Vertebrate TH synthesis occurs in the thyroid follicle and is mediated by the thyroid follicular cell. Two key functions of this cell type, iodide transport and iodide organification, are known to be inhibited by selected chemicals. Active transport of iodide into follicular cells from the blood is effected by the NIS and several chemicals are known to inhibit NIS, including the anion perchlorate (Dohan et al., 2003; van Sande et al., 2003; Wolff, 1998). Organification of iodide and the coupling of iodinated tyrosine residues are carried out by thyroid peroxidase (TPO), an apical membrane enzyme of the follicular cell, resulting in the formation of monoiodotyrosine (MIT) and diiodotyrosine (DIT) residues on thyroglobulin (TG), the substrate protein which is in high abundance in the follicular colloid. Two DIT residues are subsequently coupled by TPO to form residues of 3,5,3′,5′-tetraiodothyronine (T4) on TG. Therefore, TG normally has residues of MIT, DIT, and T4. Ultimately, T4 residues are enzymatically cleaved from TG by the follicular cell and released into general circulation. TPO is inhibitable by several chemicals, including 6-PTU and methimazole (Goos et al., 1968; Davidson et al., 1978; Nagasaka and Hidaka, 1976; Taurog, 1976).

In this study, thyroid gland MIT, DIT, and T4 all increased in the experimental controls throughout the duration of the experiment which is consistent with the expected up-regulation of TH synthesis associated with metamorphosis. Perchlorate and methimazole caused significant decreases in thyroidal MIT, DIT, and T4, while 6-PTU only reduced DIT and T4. Decreases in thyroidal MIT, DIT and T4 were evident on exposure day 2 and generally became more exaggerated on exposure days 4 and 6. Changes in these endpoints were the first to appear in this study, demonstrating that alterations of the TH synthetic pathway can be detected in advance of changes in circulating T4, thyroid histology, and thyroid cell numbers. As such, these endpoints, along with NIS, TPO, and TSHr gene expression changes (Opitz et al., 2009) are leading temporal indicators of thyroid hormone synthesis inhibition, regardless of the specific mechanism of action.

The lack of a 6-PTU effect on thyroidal MIT quantities indicates that 6-PTU may inhibit TPO differently than methimazole. These results suggest that 6-PTU inhibits TPO at the enzymatic step that leads to DIT formation, but not to MIT formation. The differential inhibition of MIT, DIT and T4 in thyroidal tissue observed here is consistent with previous *in vivo* (Richards and Ingbar, 1959; Iino et al., 1961) and *in vitro* (Engler et al., 1982) studies which evaluated the inhibitory effects of 6-PTU on TPO-mediated T4 formation. Collectively, these studies demonstrate that T4 formation was the most sensitive step in the synthetic process, followed by DIT and MIT in descending order of sensitivity.

Exposure to all three TH synthesis inhibitors reduced circulating T4 to about half of the control value by exposure day 6, lagging behind the loss of thyroidal T4 (Table 2). These observations suggest that circulating T4 concentrations are maintained temporarily through utilization of the T4 reserves present in the follicular colloid. However, once this reserve is depleted, circulating T4 concentrations are no longer maintained in the absence of T4 synthesis. By contrast, circulating T4 concentrations in the experimental controls steadily increased throughout the 6-d experiment.

All three-test chemicals caused an array of histological changes in the thyroid, including follicular cell hyperplasia in excess of controls, follicular cell hypertrophy, colloid depletion, and diffuse hypertrophy. These observations are consistent with those made in previous studies (Degitz et al., 2005; Tietge et al., 2005; Opitz et al., 2009), though this study demonstrates that effects are expressed earlier than previously reported. In general, the prevalence and severity of effects increased with time, with minimal effects observed at exposure day 4 and maximal effects observed at exposure day 8.

Depletion of colloid mass was clearly evident at the histological level, particularly in the perchlorate and methimazole treated animals, where colloid depletion led to follicular lumen collapse and the apparent loss of thyroglobulin stores. 6-PTU exposure, like perchlorate and methimazole, reduced circulating T4 concentrations and thyroidal T4 and DIT mass. But, 6-PTU exposure did not reduce thyroidal MIT concentrations, nor did it cause pervasive follicular collapse. To the contrary, 6-PTU exposure resulted in a combination of reduced colloid and selected follicular enlargement toward the end of the study. The latter observation suggesting that the retention of thyroglobulin-bound MIT residues preserve thyroglobulin stores through an unknown mechanism. The differential responses in thyroid histology observed are consistent with the observations

Table 2
Magnitude of temporal responses of several thyroid-related endpoints in larval *X. laevis* evaluated after 1, 2, 4, 6, and 8 d of exposure to methimazole, perchlorate, and 6-propylthiouracil (6-PTU). Thyroid gland MIT, DIT, and T4, serum T4, and thyroid cell numbers are significant changes expressed as percent of time-matched controls. Thyroid gland histology data are expressed as percent prevalence over mean severity. Dashes indicate no significant change; empty cells indicate no measurement.

Endpoint	Chemical	Exposure duration				
		1 d	2 d	4 d	6 d	8 d
Thyroidal MIT	Methimazole	–	51%	28%	10%	
	Perchlorate	–	–	52%	44%	
	6-PTU	–	62%	–	–	
Thyroidal DIT	Methimazole	–	47%	25%	4%	
	Perchlorate	–	53%	29%	18%	
	6-PTU	–	39%	29%	44%	
Thyroidal T4	Methimazole	–	59%	24%	6%	
	Perchlorate	–	58%	25%	14%	
	6-PTU	–	63%	14%	10%	
Circulating T4	Methimazole	–	–	–	46%	
	Perchlorate	–	–	–	54%	
	6-PTU	–	–	–	49%	
Follicular cell hyperplasia	Methimazole		80%	100%	100%	100%
			0.8	1.6	2.6	4.0
	Perchlorate		80%	100%	100%	100%
			0.8	1.4	2.0	2.6
	6-PTU		80%	100%	100%	100%
			0.8	1.6	1.4	2.8
Follicular cell hypertrophy	Methimazole		–	60%	80%	100%
			–	1.2	2.4	3.4
	Perchlorate		–	100%	100%	100%
			–	2.0	3.0	3.6
	6-PTU		20%	80%	60%	100%
			0.2	1.2	0.8	2.4
Colloid depletion	Methimazole		–	40%	80%	100%
			–	0.8	2.0	4.0
	Perchlorate		–	20%	40%	100%
			–	0.4	0.8	3.4
	6-PTU		60%	60%	60%	100%
			0.8	0.8	0.6	3.0
Diffuse thyroidal hypertrophy	Methimazole		–	–	20%	80%
			–	–	0.4	2.2
	Perchlorate		–	–	–	40%
			–	–	–	0.8
	6-PTU		–	–	20%	80%
			–	–	0.4	2.4
Follicular enlargement	Methimazole		–	–	–	–
			–	–	–	–
	Perchlorate		–	–	–	–
			–	–	–	–
	6-PTU		–	–	–	100%
			–	–	–	2.4
Thyroid cell numbers[a]	Methimazole		–	–	140%	
	Perchlorate		–	–	130%	
	6-PTU		–	–	133%	

[a] Thyroid cell numbers were also measured on exposure days 3 and 5 and no significant changes were observed.

that ETU exposure leads to follicular enlargement using a similar protocol (Opitz et al., 2009).

Diffuse hypertrophy, an observation of increased thyroid gland size due to both follicular cell hyperplasia and hypertrophy, was evident only on exposure day 8 and was consistent with the independent measurement of thyroid cell numbers, which increased significantly in all three treatments at exposure day 6. Among controls, minimal thyroid follicular cell hyperplasia was the only histological observation. This reflects the fact that the thyroid gland is normally undergoing significant growth during the pro-metamorphic period as was independently demonstrated by increases in thyroid cell numbers in the experimental controls.

Thyroid gland cell numbers were used to determine differential growth of the thyroid gland among different treatment groups and may serve as an alternative to histology as an indicator of hyperplasia. Thyroid gland cell numbers increased in the experimental controls throughout the study by about 50%, indicating that thyroid gland growth is occurring over this dynamic developmental period. Exposure to all three chemicals caused thyroid gland cell numbers to increase 30–40% over the control value on exposure day 6, a clear indication of proliferation in excess of that observed in controls. Although this measurement includes all cell types in the thyroid gland and is not specific for follicular cells, it is indicative of a general proliferative response which is consistent with the histological observations of excessive follicular cell hyperplasia and diffuse thyroidal hypertrophy in the treated animals.

Taken together, the endpoint responses evaluated in this study and others (Opitz et al., 2009) characterize both the early direct toxic effects of the test chemicals as well as compensatory responses of the HPT expressed in the thyroid gland. Reductions

in thyroidal MIT, DIT, and T4 concentrations and reductions in circulating T4 concentrations indicate that all three test chemicals inhibit T4 synthesis, as expected. This study demonstrates that these *in vivo* responses toward T4 synthesis inhibitors that operate through different mechanisms are readily detectable within a few days of exposure. Thyroid gland histology, thyroid gland cell number, and thyroid gene expression results are indicative of compensatory responses to reduced circulating T4 concentrations. The endocrinological link between reduced circulating T4 concentrations and the compensatory process is generally considered to be circulating TSH, which is released by the pituitary and upregulates T4 synthesis and release. To date, circulating TSH concentrations have not been reported in *X. laevis* larvae in either the native state or following exposure to TH synthesis inhibitors. Therefore, this link remains presumptive and is an important area for further research.

5. Conclusions

In conclusion, this work, along with that of Opitz et al. (2009), demonstrates that early temporal changes in the thyroid system are readily detectable and suggests that an alternative short term bioassay for HPT disruption is feasible. Based on the results of the experiments with perchlorate, methimazole, 6-PTU and ETU, an exposure protocol of approximately 7 d appears to be sufficient to elicit responses in all of the endpoints measured. A variety of these thyroid-related endpoints could be utilized in such an assay. But, given our current understanding of the biological system, NIS gene upregulation and thyroidal MIT, DIT, T4 concentrations are highly responsive measures with strong diagnostic linkages to the known functions of the HPT axis, particularly as they relate to TH synthesis. Changes in thyroid gland histology and thyroid cell numbers were also detected and provide a morphological linkage to compensatory mechanisms. Since these responses have been measured following exposure to relatively high concentrations of model TH synthesis inhibitors, it remains uncertain how sensitive they would be to lower concentrations of the same chemicals or to less potent thyroid hormone synthesis inhibitors. Therefore, the temporal manifestation and the relative sensitivity of these endpoints need to be further evaluated in that context.

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